Traumatic Brain Injury Impairs Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor Complex Formation and Alters Synaptic Vesicle Distribution in the Hippocampus

Shaun W. Carlson, Hong Yan, Michelle Ma, Youming Li, Jeremy Henchir, and C. Edward Dixon

Abstract

Traumatic brain injury (TBI) impairs neuronal function and can culminate in lasting cognitive impairment. While impaired neurotransmitter release has been well established after experimental TBI, little is understood about the mechanisms underlying this consequence. In the synapse, vesicular docking and neurotransmitter release requires the formation of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. Impairments in vesicle docking, and alterations in SNARE complex formation are associated with impaired neurotransmitter release. We hypothesized that TBI reduces SNARE complex formation and disrupts synaptic vesicle distribution in the hippocampus. To examine the effect of TBI on the SNARE complex, rats were subjected to controlled cortical impact (CCI) or sham injury, and the brains were assessed at 6 h, 1 d, one week, two weeks, or four weeks post-injury. Immunoblotting of hippocampal homogenates revealed significantly reduced SNARE complex formation at one week and two weeks post-injury. To assess synaptic vesicles distribution, rats received CCI or sham injury and the brains were processed for transmission electron microscopy at one week post-injury. Synapses in the hippocampus were imaged at 100k magnification, and vesicle distribution was assessed in pre-synaptic terminals at the active zone. CCI resulted in a significant reduction in vesicle number within 150 nm of the active zone. These findings provide the first evidence of TBI-induced impairments in synaptic vesicle docking, and suggest that reductions in the pool of readily releasable vesicles and impaired SNARE complex formation are two novel mechanisms contributing to impaired neurotransmission after TBI.

Key words: hippocampus; SNARE; synapse; traumatic brain injury; vesicle docking

Introduction

IMPAIRMENTS IN COGNITIVE PERFORMANCE following a traumatic brain injury (TBI) have been well described. Patients frequently cite difficulties with learning, memory, and attention that persist for years and greatly contribute to reduced quality of life.^{1–3} Hippocampal lesions, observed by magnetic resonance imaging or in post-mortem studies, and altered hippocampal function are likely contributors to post-traumatic cognitive dysfunction after TBI.^{4–7} In experimental models of TBI, markers of neuron damage and loss have been observed in multiple regions of the hippocampus, including the dentate gyrus. In the controlled cortical impact (CCI) injury model, neurodegeneration has been well-characterized throughout the hippocampal trisynaptic circuit, including the hilus, granular layer, and CA3 and CA1 fields, highlighting neurodegeneration as a contributor to impaired electrophysiological function in the hippocampus.^{8–14}

Disruptions in neurotransmitter release have been documented in the hippocampus, neocortex, and striatum in the weeks following experimental TBI by multiple methods.^{13–21} Assessments with microdialysis and high performance liquid chromatography demonstrate significant impairment in stimulated release of dopamine and acetylcholine at one week and two weeks following CCI.^{13–16,18,19} Wagner and colleagues utilized fast scan cyclic voltammetry to show that dopamine release evoked by electrical stimulation to the medial forebrain bundle is impaired in the striatum two weeks after CCI.¹⁷ Using enzyme-based microelectrode arrays, Hinzman and colleagues demonstrated that fluid percussion injury acutely elevated glutamate levels and potassium-evoked glutamate release in the striatum and dentate gyrus at 2 d, but later time-points were not assessed in these regions.^{20,21} The manifestation of neurotransmitter release deficits, independent of neurotransmitter class, in multiple brain regions with varying proximity to the injury site, suggests that a highly conserved synaptic mechanism may underlie these pathologies.

Regulated fusion of a synaptic vesicle with the plasma membrane and the subsequent release of neurotransmitters into the synaptic cleft are critical for neuronal communication. Vesicular fusion in the presynaptic terminal is dependent upon the availability of vesicles and the formation of the molecular machinery that catalyzes membrane fusion. Reductions in the number of vesicles in the readily releasable pool, or vesicles immediately available upon stimulation, are associated with impaired neurotransmission.^{22–26} In the presynaptic terminal, formation of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex is a crucial step to initiate vesicle docking and fusion with the plasma membrane.²⁷ The SNARE complex is comprised of multiple proteins, including synaptobrevin 2 (VAMP2) and cysteine string protein alpha (CSP α) on the vesicular membrane, and syntaxin-1 and synaptosomal-associated protein of 25 kDa (SNAP-25) on the plasma membrane. Reductions in the abundance of CSP α , SNAP-25, or VAMP2 by genetic manipulation significantly impair SNARE complex formation, vesicle docking, and neurotransmitter release.^{28–31}

The packing density of synapses in CA-1 of the dentate gyrus is significantly reduced to approximately 60% within 48 h of CCI, and recovers to approximately 50% synaptic packing density at 10 d post-injury, as assessed by ultrastructural imaging.³² While CCI has been shown to reduce synaptic density and the abundance of synaptic markers in the hippocampus,^{8,32,33} little is known about the intrasynaptic vesicle properties of the surviving hippocampal synapses following TBI. In the current study, we examined the effect of TBI on intrasynaptic vesicle properties in the hippocampus. The abundance of monomeric SNARE proteins and assembled complexes were evaluated by immunoblotting, and changes in synaptic vesicle number and distribution were evaluated using ultrastructural imaging. The abundance of assembled SNARE complexes was significantly reduced at one week and two weeks post-injury. CCI resulted in a significant reduction in vesicle frequency within 150 nm of the active zone of the synaptic terminal at one week post-injury, a time-point associated with reduced neurotransmitter release. These findings suggest that altered synaptic vesicle distribution and impaired vesicular docking are two novel mechanisms that may contribute to impaired neurotransmission and potentially hippocampal-dependent cognitive dysfunction after TBI.

Methods

Animals

All experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee in accordance with the guidelines established by the National Institutes of Health in the Guide for the Care and Use of Laboratory Animals. Animals were housed up to two rats per cage in the University of Pittsburgh vivarium with a 12:12 light/dark photoperiod and provided food and water ad libitum. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300–350 g were used in this study.

CCI

Animals were randomly assigned to receive either sham or CCI injury. SNARE protein abundance was evaluated by immunoblotting at 6 h, 1 d, one week, two weeks, and four weeks post-injury, and SNARE protein localization was visualized by immunohistochemistry at two weeks post-injury. Changes in SNARE protein levels have not been evaluated after TBI, and in order to assess temporal alterations, we selected multiple acute and chronic time-points post-injury ($n=6$ per injury condition at each time-point). The time-course of 6 h to four weeks post-injury was selected to

assess SNARE protein abundance at time-points preceding and subsequent to established impairments in hippocampal neurotransmitter release after CCI.^{13–15} Additionally, the time-course was designed to evaluate SNARE protein changes at time-points similar to the temporal progression of CA1 subfield synaptic density reductions and recovery described after CCI.³² Based upon the SNARE protein immunoblot findings and the onset of impaired neurotransmission,¹⁴ ultrastructural intrasynaptic vesicular properties were evaluated by transmission electron microscopy at one week post-injury ($n=6$ per injury condition), a time-point with established deficits in evoked acetylcholine and dopamine release after CCI.^{14,18,19} Rats were anesthetized using 4% isoflurane with a 2:1 N₂O/O₂ mixture in a ventilated anesthesia chamber. Following endotracheal intubation, the rats were mechanically ventilated with a 1–1.5% isoflurane mixture. Animals were placed in a stereotaxic frame in the supine position and body temperature was monitored by rectal thermistor probe and maintained at 37°C with a heating pad. Following a midline incision, the soft tissues were reflected and a 7 mm craniectomy was completed between bregma and lambda and centered 5 mm lateral of the sagittal suture to expose the dura mater over the right parietal cortex. Control sham injury animals were subjected to anesthesia and surgical procedures, but did not receive a TBI. The CCI injury device is a small bore (1.975 cm) double-acting stroked-constrained pneumatic cylinder with a 5.0 cm stroke. An impactor tip (6 mm in diameter) was set to produce a tissue deformation of 2.7 mm at a velocity of 4 m/sec with a dwell time of 150 msec. Following sham or CCI injury, the scalp was sutured, anesthesia stopped, and the righting time of each animal was monitored and recorded. Once ambulatory, the animals were returned to their home cage.

Brain tissue preparation for western blot analysis

A total of 12 rats ($n=6$ for each injury condition at each time-point) received an overdose of Fatal-plus (intraperitoneally, 100 mg/kg sodium pentobarbital, Vortech Pharmaceuticals, Dearborn, MI) and were euthanized at 6 h, 1 d, one week, two weeks, or four weeks following sham or CCI injury. After removal of the brain, the ipsilateral hippocampus was rapidly dissected on a chilled ice plate, immediately frozen in liquid nitrogen and stored at –80°C. Samples were homogenized in lysis buffer (0.1 M NaCl, 0.01 M Tris-Cl, 0.001 M EDTA, pH 7.6) with protease inhibitor cocktail kit (Pierce, Rockford, IL). The homogenized samples were centrifuged at 12,000 $\times$ g at 4°C for 10 min and the supernatants were collected. The protein concentration was determined by a BCA protein assay kit (Thermo Scientific, Pittsburgh, PA) using a 96-well microplate reader (Biotek, Winooski, VT).

Brain tissue preparation for immunohistochemistry

A total of 12 rats ($n=6$ for each injury condition) received an overdose of Fatal-plus (intraperitoneally, 100 mg/kg sodium pentobarbital) at two weeks following sham and CCI injury. Animals were transcardially perfused with saline, followed by a mixture of 10% neutral buffered formalin (Fisher Scientific, Waltham, MA). The brains were post-fixed for an additional 24 h in 10% neutral buffered formalin, and subsequently cryoprotected with 30% sucrose in 0.1 M phosphate buffered saline (PBS) for 48 h at 4°C. The brains were frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and cut into 35 μ m thick coronal sections on a cryostat (Leica Microsystems Inc., Buffalo Grove, IL). Sections between –2.5 mm and –5.0 mm bregma were selected and processed for immunofluorescence staining.

Brain tissue preparation for transmission electron microscopy

A total of 12 rats ($n=6$ for each injury group) received an overdose of Fatal-plus (100 mg/kg sodium pentobarbital, intraperitoneally) at

one week following sham or CCI injury. Animals were transcardially perfused by gravity feed with saline, followed by a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brains were removed and placed in cold infusate for an additional 24 h. A 1 mm cubic block of tissue was dissected from the ipsilateral hippocampus to include the CA stratum radiatum (Rad), stratum lacunosum moleculare (LMol) and the molecular layer dentate gyrus (MoDG; Fig. 5A).³⁴ The tissue block was dissected at approximately -4 mm bregma, the epicenter of injury. Tissue blocks were post-fixed with 1% osmium tetroxide in 0.1 M sodium phosphate buffer, stained with uranyl acetate, and cut at a thickness of 70 nm. Six randomly selected sections were used for imaging and quantification.

Immunoblotting

To evaluate SNARE protein monomers, hippocampal homogenates were boiled for 10 min prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SNARE complex formation was assessed in unboiled protein samples to retain high molecular weight complexes, and were defined as SNAP-25 and syntaxin immunoreactive material greater than 50 kDa that was absent from boiled samples as previously described.^{29,30,35-38} Protein samples (20 μ g) and molecular weight markers (Bio-Rad, Hercules, CA) were separated using SDS-PAGE. The resolved proteins were electrophoretically transferred to a polyvinylidene fluoride membrane. The blots were incubated in 5% nonfat dry milk in tris-buffered saline (blocking solution) for 1 h. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C. The primary antibodies utilized were: goat anti-SNAP-25 (Millipore, Billerica, MA), rabbit anti-Syntaxin (Life Span Bioscience Inc., Seattle, WA), rabbit anti-CSP alpha (Abcam, Cambridge, MA), rabbit anti-VAMP2 (Life Span Bioscience Inc.). The following day, the membranes were washed with 1×TBS buffer, incubated in blocking solution for 1 h, and incubated for 1 h with directly conjugated horse radish peroxidase secondary antibodies diluted in blocking solution. The secondary antibodies utilized were goat anti-mouse, goat anti-rabbit, and donkey anti-goat horse-radish peroxidase conjugated antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Proteins were visualized with a chemiluminescence detection system (Supersignal; Pierce). To confirm equal loading, the membranes were stripped and reprobed with mouse anti- β actin monoclonal antibody (Sigma, St. Louis, MO). Sham and CCI brain-injured samples were loaded in the same gel for comparison. Blots were exposed to autoradiographic x-ray film between 10 sec to 5 min and the optic density was quantified using SCION ImagePC (Frederick, MD) software. Values are presented as the ratio of optic densities of injured samples as a percentage of sham control for each time-point.

Immunofluorescence staining

Confocal microscopy was performed to evaluate changes in SNARE protein immunoreactivity after CCI. A two week time-point was selected for immunohistochemistry as reductions were observed in multiple SNARE proteins at the two week time-point by immunoblot. Immunohistochemical staining was completed in 24-well culture plates on free-floating tissue sections. At least four sections per animal spanning the hippocampus were rinsed with 0.1 M PBS buffer and blocked with 10% normal goat serum and 0.1% Triton X-100 in 0.1 M PBS for 1 h. Sections were incubated overnight at 4°C with the same rabbit anti-CSP alpha (Abcam), rabbit anti-VAMP2 (Life Span Bioscience Inc.), and goat anti-SNAP-25 (Millipore) antibodies utilized for immunoblot analysis. On the following day, sections were rinsed with 0.1 M PBS and incubated with secondary antibodies directly conjugated with Alexa Fluor 488 and 594 fluorophores (Jackson ImmunoResearch,

Inc.) for 1 h. Labeled sections were rinsed with 0.1 M PBS, incubated with DAPI (Vectashield; Vector Laboratories, Burlingame, CA) mounted on Superfrost slides (Fisher Scientific), and stored at 4°C. Control experiments, in which primary antibodies were omitted, were completed in parallel to confirm antibody specificity. Images were acquired on a C2 confocal microscope (Nikon, Melville, NY). Representative images in the figures are maximum intensity projection images collected as a z stack (1.5 μ m step size) at 20× magnification.

Transmission electron imaging and quantification

For each animal, 20 randomly selected synapses were imaged in six sections using a transmission electron microscope (JEOL 1011; Peabody, MA). The methodology for quantification was based upon previously published reports evaluating synaptic vesicular properties.^{23-26,39-42} The synapses were imaged at 100,000× magnification and the acquired images were utilized for quantification. To examine vesicle number, the area within 450 nm of the active zone was defined and the total number of vesicles was manually counted in each imaged synapse using Metamorph software (Molecular Devices, Sunnyvale, CA). The length of the active zone, defined by a thickened presynaptic membrane with electron dense material in alignment with the postsynaptic membrane, was measured in each synapse using Metamorph software. Mean vesicle number and active zone length values were calculated for each animal and used to calculate the average value for the injury group. To assess vesicular distribution, the shortest distance between the center of a vesicle and the active zone was measured for every vesicle, within 250 nm of the active zone, with Metamorph software. Vesicles smaller than 25 nm were excluded to prevent inclusion of microtubules, which have a cross-sectional diameter of 20 nm and could not be differentiated from vesicles in the electron micrographs.²⁴ To evaluate alterations in vesicle number as a function of distance, 50 nm bins were designated and used to determine changes in vesicle frequency in 50 nm intervals from the active zone. The bin for distance 0-50 nm encompasses vesicles that were in the fusion or docking process, or in a position primed for docking. A mean vesicle frequency for each 50 nm bin was determined for each animal, and used to calculate the average vesicle frequency in that distance bin for that group.

Statistical analyses

All quantification was completed by an investigator blinded to the injury conditions of each animal. Data are presented as mean $\pm$ standard error of the mean (SEM). Quantification of total vesicle number and active zone length were compared using a Student's t -test. A Kolmogorov-Smirnov test was used to compare the cumulative distribution of synaptic vesicle frequency. A repeated measures one-way analysis of variance (ANOVA) followed by a Bonferroni post hoc t -test was used to compare synaptic vesicle frequency as a function of distance from the active zone. Immunoblot data for each SNARE protein were compared by two-way ANOVA followed by a Bonferroni post hoc t -test, when appropriate. Statistical tests were completed using Graphpad (Graphpad, La Jolla, CA). A p value of less than 0.05 was considered statistically significant for all comparisons.

Results

Synaptic membrane SNARE monomeric protein abundance is altered after CCI

To examine if SNARE proteins on the pre-synaptic membrane change after CCI, the abundance of SNAP-25 and syntaxin-1 in the hippocampus was assessed at multiple time-points after CCI. The abundance of SNAP-25 was significantly increased by 42% at one week after CCI, compared with sham injury ($p < 0.01$, post hoc

t-test following two-way ANOVA; overall time, and interaction effects, $p < 0.01$; Fig. 1A). However, the abundance of SNAP-25 was significantly reduced by 21% at two weeks, compared with sham injury ($p < 0.05$). The abundance of syntaxin was unchanged at all time-points assessed (Fig. 1B).

Vesicular membrane SNARE protein abundance is reduced after CCI

To evaluate the effect of TBI on SNARE proteins located on vesicular membranes, the abundance of VAMP2 and the chaperone protein CSP α were examined in the hippocampus in the hours, days, and weeks after CCI. The abundance of VAMP2 was significantly reduced by 29% at one week and by 33% at four weeks after brain injury ($p < 0.01$, post hoc *t*-tests following two-way ANOVA; overall injury, time, and interaction effects, $p < 0.01$), compared with sham injury at each respective time-point (Fig. 2A). The level of VAMP2 was reduced at two weeks after CCI, but this was not significantly different from sham injury.

FIG. 1. Controlled cortical impact (CCI) altered monomeric abundance of the synaptic plasma membrane protein synaptosomal-associated protein of 25 kDa (SNAP-25). (A) Representative western blot image for SNAP-25 in the hippocampus at two weeks (WK) post-injury in sham and CCI brain-injured rats. The abundance was determined by semi-quantitative western blot analysis. CCI resulted in an increase in SNAP-25 at 1 WK ($\#p < 0.01$) and a reduction at 2 WK ($*p < 0.05$, post hoc *t*-tests following two-way analysis of variance) post-injury, compared with sham-injury at each respective time-point. (B) Representative western blot image for syntaxin (35 kDa) in the hippocampus at 2 WK in sham-injured and brain-injured rats. Semi-quantitative assessment revealed that CCI did not alter syntaxin abundance at any time-point assessed ($n = 6$ sham-injured and $n = 6$ brain-injured rats).

FIG. 2. Controlled cortical impact (CCI) reduced monomeric abundance of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins on the vesicle membrane. (A) Representative western blot image for synaptobrevin 2 (VAMP2; 18 kDa) in the hippocampus at four weeks (WK) post-injury in sham and CCI brain-injured rats. Assessment by semi-quantitative Western blot analysis revealed that CCI injury resulted in a reduction in VAMP2 protein abundance at 1 WK and 4 WK ($\#p < 0.01$, post hoc *t*-tests following two-way analysis of variance [ANOVA]) post-injury, compared with sham surgery at each respective time-point. (B) Representative western blot images for cysteine string protein alpha (CSP α ; 34 kDa) in the hippocampus at 1 WK in sham-injured and CCI brain-injured rats. Semi-quantitative assessment revealed that CCI significantly reduced CSP α abundance at 1 d (D) and 1 WK ($*p < 0.05$, post hoc *t*-tests following two-way ANOVA) post-injury compared with sham injury at each respective time-point ($n = 6$ sham-injured and $n = 6$ brain-injured rats).

The abundance of CSP α was significantly reduced by 20% at 1 d and 26% at one week after CCI ($p < 0.05$, post-hoc *t*-tests following two-way ANOVA, overall injury, time, and interaction effects: $p < 0.01$; Fig. 2B), compared with sham injury at each respective time-point. The levels of CSP α began to recover to sham-injury levels starting at two weeks after CCI. The reduction in CSP α abundance at 1 d post-injury preceded CCI-induced alterations in the abundance of SNAP-25 and VAMP2 proteins.

SNARE protein alterations in the hippocampus after CCI

In order to visualize SNARE proteins in the injured hippocampus, we utilized immunohistochemistry to determine if alterations in SNAP-25, VAMP2, and CSP α immunoreactivity could be visualized at two weeks following CCI. Assessments of SNARE protein immunoreactivity were completed in the CA3 field of the

FIG. 3. Controlled cortical impact (CCI) altered immunoreactivity for soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins in the hippocampus at two weeks post-injury. Representative images of immunoreactivity for synaptosomal-associated protein of 25 kDa (SNAP-25), synaptobrevin 2 (VAMP2), and cysteine string protein alpha (CSPα) in the ipsilateral hippocampus at two weeks post-injury. (A) Adapted image of a stereotaxic plate diagram³⁴ depicting the hippocampal layers in which SNARE immunoreactivity was observed (black box). (B) Representative images of alterations in SNAP-25 immunoreactivity in the stratum lucidum (SLu) and the pyramidal cell layer (Py) in the hippocampus of sham and CCI brain-injured rats. (C) CCI injury resulted in an observable reduction in punctate synaptic-like staining of VAMP2 in the stratum radiatum (Rad), SLu and Oriens (Or) layers at two weeks post-injury. (D) CCI resulted in an apparent reduction in the density of punctate CSPα staining in stratum Rad and SLu layers at two weeks post-injury, compared with sham injury. White arrows indicate examples of representative staining for each respective marker in panels B-D. Scale bar represents 50 μm ($n=6$ sham-injured and $n=6$ brain-injured rats).

hippocampus, as indicated by the inset black box in Figure 3A on an adapted image of a stereotaxic plate diagram at -4 mm bregma.³⁴ SNARE protein immunoreactivity was observed, primarily in the stratum radiatum (Rad) and stratum lucidum (SLu). While the immunoreactivity appeared primarily in the SLu layer, without additional staining to delineate the Rad and SLu layers the precise anatomical location of the immunopositive SNARE proteins cannot be defined. SNAP-25 immunoreactivity appeared fiber-like and could reflect labeling of projections located in the SLu layer and pyramidal cell layer (Py; arrows, Fig. 3B). Following CCI, there was an observable reduction in hippocampal SNAP-25 immunoreactivity with reduced projection staining intensity, and an apparent decrease in the number of SNAP-25-positive projections.

In sections from sham-injured rats, VAMP2 immunoreactivity was characterized by punctate staining, consistent with synaptic staining, in the Rad, SLu, and Oriens (Or) layers of the hippocampus (arrows, Fig. 3C). CCI resulted in a visible reduction in VAMP2 immunoreactivity in the same layers. Similar to VAMP2, CSPα immunoreactivity was observed as a punctate synaptic-like pattern in the SLu and Or layers in sham-injured rats (arrows, Fig. 3D). However, after CCI, there was an observable reduction in the density of punctate CSPα staining in the SLu layer. Collectively, these images corroborate our semiquantitative western blot findings that CCI results in a reduction in SNARE proteins in the hippocampus at two weeks post-injury.

Assembly of the SNARE complex is impaired after CCI

Reductions in the formation of the SNARE complex is associated with impaired synaptic vesicle docking and fusion to the plasma membrane. SNARE complex formation was assessed by measuring SNAP-25 and syntaxin immunoreactive high molecular weight complexes greater than 50kDa in hippocampal homogenates. The abundance of SNAP-25 immunoreactive complexes was reduced by 58% and 61% at one week and two weeks post-injury, respectively, compared with sham-surgery ($p<0.01$, post hoc t -tests following two-way ANOVA; overall injury, time, and interaction effects, $p<0.01$, Fig. 4A). Similarly, evaluation of syntaxin immunoreactive SNARE complexes in CCI brain-injured rats exhibited a 50% and 41% reduction in SNARE complex formation at one ($p<0.05$) and two weeks post-injury, respectively, compared with sham-injured rats ($p<0.01$, post hoc t -tests following two-way ANOVA; overall injury, time, and interaction effects, $p<0.01$, Fig. 4B). The time-course for CCI-induced alterations in monomeric SNARE proteins and SNARE complexes vary for individual SNARE proteins, but alterations in the abundance of multiple proteins and reduced complex formation were mainly observed at one week and two weeks post-injury.

Synaptic vesicle distribution is altered after CCI

To evaluate if synaptic vesicular pool distribution is altered at one week following CCI, the distribution of vesicles in proximity to the pre-synaptic active zone were examined by ultrastructural electron microscopy in the CA stratum Rad, CA stratum LMol, and the molecular layer of the dentate gyrus (Fig. 5A). In nerve terminals of sham-injured rats, synaptic vesicles qualitatively appeared distributed around the active zone, with the highest frequency within approximately 250 nm of each active site (Fig. 5B). Comparatively, synapses from CCI brain-injured rats exhibited reduced vesicle number around the active site, and reduction in the number of vesicles in the readily releasable pool, as identified within 50 nm of the active zone. Quantification of vesicle

FIG. 4. Controlled cortical impact (CCI) reduced formation of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex at 1 week (WK) and 2 WK post-injury. **(A)** Representative western blot image for synaptosomal-associated protein of 25 kDa (SNAP-25) immunoreactive SNARE complexes in the hippocampus at 2 WK post-injury in sham and CCI brain-injured rats. Assessment by semi-quantitative western blot analysis revealed that CCI resulted in a reduction in complex abundance at 1 and 2 WK post-injury ($\#p < 0.01$, post hoc *t*-tests following two-way analysis of variance [ANOVA]), compared with sham-injury at each respective time-point. **(B)** Representative Western blot image for syntaxin immunoreactive SNARE complexes in the hippocampus at 2 WK post-injury in sham and brain-injured rats. Semi-quantitative Western blot analysis revealed a significant reduction in syntaxin complex abundance at 1 WK ($*p < 0.05$) and 2 WK post-injury ($\#p < 0.01$, post hoc *t*-tests following two-way ANOVA), compared with sham-injury at each respective time-point ($n = 6$ sham-injured and $n = 6$ brain-injured rats).

number revealed a significant reduction ($p < 0.05$, Student's *t*-test) in vesicle number following CCI (53.7 ± 4 vesicles), compared with sham injury (80.6 ± 10 vesicles; Fig. 5C). However, no difference in active zone length was observed between CCI-injured (296 ± 8 nm) and sham-injured (288 ± 7 nm) groups (Fig. 5D).

In order to examine changes in presynaptic vesicle distribution after CCI, the shortest distance of each individual vesicle from the active site was measured. Consistent with the qualitative observation, CCI-injured rats exhibited a significant alteration in the distribution of synaptic vesicles within 250 nm of the active zone

($p < 0.05$, Kolmogorov-Smirnov test). Comparison of vesicle number as a function of distance revealed that CCI reduced vesicular frequency within 50 nm, and in the vesicular pool up to 150 nm from the active zone, compared with sham-injured rats ($\#p < 0.001$; $*p < 0.05$; post hoc *t*-tests following repeated measures one-way ANOVA; overall injury effect, $p < 0.05$; Fig. 5E). CCI injury resulted in an approximate 50% reduction in vesicle frequency within 50 nm, and 20% reduction between 50 and 150 nm of the active zone, compared with sham injury. Vesicle frequency was not significantly different between groups at distances greater than 150 nm from the active zone.

Discussion

The goal of the current study was to provide additional lines of evidence to the hypothesis that changes in synaptic vesicle properties, including vesicle distribution and the proteins involved in vesicle docking at the active zone, could contribute to impaired neurotransmission after TBI. We demonstrate for the first time that TBI disrupts the distribution of synaptic vesicles in pre-synaptic terminals in the injured hippocampus. Our findings describe TBI-induced impairments in the assembly of the machinery critical for vesicular fusion at the synaptic membrane. CCI produced significant alterations in the abundance of monomeric SNARE proteins and formation of the SNARE complex formation in the hippocampus. Previous work from our lab demonstrates that CCI reduces scopolamine-evoked acetylcholine release in the hippocampus at one week and two weeks after brain injury.^{13–15} The manifestation of altered vesicle distribution at one week post-injury and reduced abundance of SNARE complexes at one week and two weeks post-injury suggests that alterations in the highly-conserved SNARE protein complex may contribute to impaired neurotransmitter release after TBI.

Neurotransmission is a highly regulated process that requires vesicular docking at the active zone to facilitate the release of neurotransmitters into the synaptic cleft. Reductions in the readily releasable vesicle pool, vesicles within 50 nm of the active zone, can impair the release of neurotransmitters.^{22,40} Our analysis of electron micrographs revealed that TBI resulted in a significant reduction in vesicle number and reduced vesicle frequency within 150 nm of the active zone of a presynaptic terminal at one week following CCI. The finding that the active zone length was not altered after TBI suggests the reduced vesicular number is not reflective of changes in active zone size. Reductions in vesicular frequency within 50 nm of the active zone is associated with reduced docking,²² and the decreased vesicular number observed within 150 nm of the active zone could be indicative of impaired vesicular priming or translocation of vesicles to the synaptic membrane.

To the best of our knowledge, this is the first work to demonstrate that TBI disrupts synaptic vesicle distribution. Scheff and colleagues³² previously showed, by ultrastructural analysis, that CCI produces a significant reduction in CA1 subfield stratum Rad synaptic density up to two months post-injury. A maximal reduction was observed at 2 d post-injury, followed by a gradual recovery to approximately 70% of sham levels by 60 d post-injury. In our study, we demonstrate that extant hippocampal synapses exhibit altered vesicular number and distribution. Scheff and colleagues also reported that CCI did not alter synaptic contact length.³² Comparatively, we found that the active zone lengths were similar between sham and CCI brain-injured groups and similar to the values reported by Scheff and colleagues, suggesting that the vesicular disruptions were not the result of altered synaptic contacts.

FIG. 5. Controlled cortical impact (CCI) altered vesicular distribution in the presynaptic nerve terminal at one week post-injury. (A) Adapted image of a stereotaxic plate diagram³⁴ depicting the hippocampal layers in which synaptic vesicle properties were evaluated (black box). (B) Representative transmission electron micrographs of presynaptic nerve terminals (NT) in the hippocampus of sham and CCI brain-injured rats. Vesicular distribution around the presynaptic active site (white arrow) appeared altered in brain-injured rats, compared with sham rats at 7 d post-injury. Scale bar represents 100 nm. Dendritic spine (Sp). (C) CCI injury resulted in a significant reduction in the number of synaptic vesicles at one week post-injury, compared with sham injury (* $p < 0.05$, Student's t -test). (D) Active zone length was not different between sham injury and CCI injury. (E) Evaluation of vesicular frequency as a function of distance demonstrates that CCI produced a significant reduction in vesicle number within 150 nm of the presynaptic active site, compared with sham-injury (# $p < 0.01$, * $p < 0.05$, post hoc t -tests following repeated measures one-way analysis of variance). Vesicle number, active zone length, and vesicular frequency were assessed in 20 randomly selected presynaptic terminals for each animal ($n = 6$ sham-injured and $n = 6$ brain-injured rats).

The reduction and slow recovery of synaptic packing density described between 2 and 15 d is reflective of both synaptic loss and synaptogenesis.³² Morphological assessment of dendritic spines at 3 d following CCI reveals significant reductions in the numbers of mushroom-like and filopodia-like spines,⁸ morphology indicative of mature or transitional spines, respectively, possibly undergoing retraction.⁴³ The assessment of synaptic vesicle number and distribution in the current study was completed in hippocampal synapses with clearly defined active zones. However, we were not able to delineate potential differences between mature synapses, degenerating synapses, or the establishment of newly-formed synapses. Additional studies are needed to better understand if altered vesicle distribution correlates with synaptic maturity or indicators of synaptic damage.

Altered synaptic vesicle distribution could be a consequence of impaired vesicular translocation and docking at the synaptic membrane. The proteins comprising the SNARE complex are highly conserved and are an important component of exocytosis, including facilitating regulated neurotransmitter release in the synaptic cleft.^{27,44} VAMP2, SNAP-25, syntaxin-1, and CSP α represent key vesicular and synaptic membrane SNARE proteins, and a key chaperone protein in the SNARE complex, respectively. Reductions in the levels of these key SNARE proteins are associated with impaired SNARE complex formation and reduced ve-

sicular fusion. Cultured neurons from heterozygous VAMP2 knockout mice exhibited significantly reduced synaptic vesicle fusion during hypertonic sucrose-induced and Ca^{2+} -triggered fusion.³¹ Targeted knockout of CSP α resulted in both reduced SNAP-25 abundance and SNARE complex assembly.²⁸ In CSP α knockout mice, reductions in CSP α were associated with reduced SNAP-25 abundance, and increased ubiquitin-dependent degradation of SNAP-25.²⁹ Moreover, targeted knockdown of SNAP-25 in CSP α knockout mice resulted in exacerbated deficits in SNARE complex formation.²⁹ Collectively, these mechanistic studies support the hypothesis that reductions in key SNARE protein monomers and/or chaperone proteins could reduce SNARE complex formation and contribute to impaired neurotransmission after TBI.

Little is known about the effect of TBI on synaptic proteins that comprise the SNARE protein complex. Complexin I, a chaperone protein that binds to SNAP-25, was shown to be transiently increased at 6 h and decreased between 3 and 7 d in the cortex, and decreased at only 7 d post-injury in the hippocampus following fluid percussion injury.⁴⁵ The reduction in complexin I levels at one week post-injury in the hippocampus is consistent with reductions in other SNARE proteins observed in the current study. We show that CCI significantly altered the monomeric levels of CSP α , SNAP-25, and VAMP2. CSP α was the only SNARE protein to be significantly reduced at 1 d, and the decreased abundance extended

to one week post-injury. The abundance of SNAP-25 showed a transient elevation at one week followed by reduced abundance at two weeks post-injury. Targeted knockout of CSP α is associated with a significant reduction in SNAP-25 abundance, increased degradation of SNAP-25, and impaired SNARE complex formation.^{28,29} The transient increase in SNAP-25 observed at one week post-injury could be reflective of altered SNAP-25 degradation, a process in which CSP α has been implicated as an important component.^{29,30} The reductions in VAMP2 at one week and four weeks post-injury highlight that alterations in SNARE proteins can persist for weeks post-injury.

It is unknown if the reductions in SNAP-25 or the other SNARE proteins after CCI are reflective of post-translational modifications that increase proteasomal degradation, which have been reported under normal physiological conditions.²⁹ Increased protease activity after CCI may contribute to reductions in SNARE proteins, as SNAP-25 has been identified as a substrate for calpain after TBI.⁴⁶ It is also plausible that the reduction in monomeric levels could be reflective of decreased gene expression, as central nervous system-specific GeneChip data analysis of the adult rat cerebral cortex at 24 h after CCI revealed a significant reduction in SNAP-25 transcripts.⁴⁷ In our study, we observed no change in SNAP-25 protein levels at 1 d post-injury. This difference could be reflective of proximity to the contusion site, as our study assessed hippocampal, and not cortical, protein abundance. The mechanisms by which SNARE proteins are reduced after TBI are poorly understood, and future work is warranted to better understand the effect of TBI on SNARE protein expression, stability, and degradation.

An alternative explanation is that synaptic loss accounts for the reduction in monomeric SNARE protein abundance after CCI. However, the ultrastructural data suggests that underlying deficits in vesicle dynamics persist in established synapses in the injured hippocampus. Additionally, if the loss of SNARE proteins was reflective of reduced synaptic density, a maximal reduction in SNARE proteins would be predicted around 2 d post-injury, with a gradual increase during the four weeks post-injury, as suggested by the ultrastructural analysis of synaptic density.³² Our semi-quantitative data shows that reductions in CSP α occur at 1 d, while reductions in other SNARE proteins are delayed and persist for weeks post-injury. Additionally, we observe no alterations in the level of syntaxin at any time-point assessed after CCI. Reductions in synaptophysin abundance, as assessed by semi-quantitative analysis, have been reported within one week following CCI,^{8,48} while another report demonstrates transient alterations in synaptophysin abundance post-injury.³³ These differences may be reflective of injury severity, species utilized, and the region in which protein levels were assessed. Taken together, it is probable that reductions in SNARE proteins and complex formation are reflective of intrasynaptic changes and not purely reflective of synaptic loss in the injured hippocampus.

The finding that CCI reduced monomeric SNARE protein levels, suggested that SNARE complex formation also would be reduced in the injured brain. As posited, CCI resulted in a significant reduction in SNARE complex formation at one week and two weeks post-injury using two different markers for assembled complexes. Immunoblotting for high-molecular weight SNAP-25 and syntaxin positive complexes revealed robust reductions in the abundance of assembled SNARE complexes. This is the first evidence that demonstrates that TBI significantly impairs the synaptic machinery responsible for vesicle docking and membrane fusion. Considering reductions in SNARE complex formation are associated with impairments in vesicle docking and neurotransmitter release into the

synaptic cleft, these findings provide exciting evidence that impaired SNARE complex formation contribute to deficits in neurotransmission after TBI.

In summary, we demonstrate for the first time that CCI produces a significant alteration in synaptic vesicle distribution at one week post-injury. Additionally, CCI significantly alters the abundance of SNARE proteins and reduces the formation of the SNARE complex, the machinery critical for synaptic vesicle docking. The findings of altered synaptic vesicle distribution and impaired SNARE complex formation suggest that alterations in synaptic vesicle docking may be a common underlying mechanism that contributes to deficits in neurotransmission in multiple brain regions and in different neurotransmitter systems of the injured brain. This study provides novel evidence that altered vesicle distribution and impaired vesicle docking may be novel contributors to deficits in neurotransmission after TBI.

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Author Disclosure Statement

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Address correspondence to:

C. Edward Dixon, PhD

Department of Neurosurgery

Safar Center for Resuscitation Research

Pittsburgh VA Healthcare System

3434 Fifth Avenue

Pittsburgh, PA 15260

E-mail: dixonec@upmc.edu